including hybridization, biotin incorporation with streptavidin capture, or by virtue if the fact that the longer molecules bind more predictably and efficiently to a number of nucleic acid minding matrices, such as nitrocellulose, nylon, or glass, in membrane, paper, resin, or other form. While not required for this assay, this separation of functions allows effective exclusion from signal of both unreacted probe and tailed target nucleic acid.

In addition to the supports decribed above, the tailed products may be captured onto any support that contains a suitable capture moiety. For example, biotinylated products are generally captured with avidin-treated surfaces. These avidin surfaces may be in microtitre plate wells, on beads, on dipsticks, to name just a few of the possibilities. Such surfaces can also be modified to contain specific oligonucleotides, allowing capture of product by hybridization. Capture surfaces as described here are generally known to those skilled in the art and include nitrocellulose dipsticks (*e.g.*, GeneComb, BioRad, Hercules, CA).

VIII. Improved Enzymes For Use In Invader™-Directed Cleavage Reactions

A cleavage structure is defined herein as a structure which is formed by the interaction of a probe oligonucleotide and a target nucleic acid to form a duplex, the resulting structure being cleavable by a cleavage means, including but not limited to an enzyme. The cleavage structure is further defined as a substrate for specific cleavage by the cleavage means in contrast to a nucleic acid molecule which is a substrate for nonspecific cleavage by agents such as phosphodiesterases. Examples of some possible cleavage structures are shown in Figure 16. In considering improvements to enzymatic cleavage means, one may consider the action of said enzymes on any of these structures, and on any other structures that fall within the definition of a cleavage structure. The cleavage sites indicated on the structures in Figure 16 are presented by way of example. Specific cleavage at any site within such a structure is contemplated.

Improvements in an enzyme may be an increased or decreased rate of cleavage of one or more types of structures. Improvements may also result in more or fewer sites of cleavage on one or more of said cleavage structures. In developing a library

of new structure-specific nucleases for use in nucleic acid cleavage assays, improvements may have many different embodiments, each related to the specific substrate structure used in a particular assay.

As an example, one embodiment of the Invader™-directed cleavage assay of the present invention may be considered. In the Invader™ directed cleavage assay, the accumulation of cleaved material is influenced by several features of the enzyme behavior. Not surprisingly, the turnover rate, or the number of structures that can be cleaved by a single enzyme molecule in a set amount of time, is very important in determining the amount of material processed during the course of an assay reaction. If an enzyme takes a long time to recognize a substrate (*e.g.*, if it is presented with a less-than-optimal structure), or if it takes a long time to execute cleavage, the rate of product accumulation is lower than if these steps proceeded quickly. If these steps are quick, yet the enzyme "holds on" to the cleaved structure, and does not immediately proceed to another uncut structure, the rate will be negatively affected.

Enzyme turnover is not the only way in which enzyme behavior can negatively affect the rate of accumulation of product. When the means used to visualize or measure product is specific for a precisely defined product, products that deviate from that definition may escape detection, and thus the rate of product accumulation may appear to be lower than it is. For example, if one had a sensitive detector for trinucleotides that could not see di- or tetranucleotides, or any sized oligonucleotide other that 3 residues, in the iIvader™-directed cleavage assay of the present invention any errant cleavage would reduce the detectable signal proportionally. It can be seen from the cleavage data presented here that, while there is usually one site within a probe that is favored for cleavage, there are often products that arise from cleavage one or more nucleotides away from the primary cleavage site. These are products that are target dependent, and are thus not non-specific background. Nevertheless, if a subsequent visualization system can detect only the primary product, these represent a loss of signal. One example of such a selective visualization system is the charge reversal readout presented herein, in which the balance of positive and negative charges determines the behavior of the products. In such a system the presence of an

extra nucleotide or the absence of an expected nucleotide can excluded a legitimate cleavage product from ultimate detection by leaving that product with the wrong balance of charge. It can be easily seen that any assay that can sensitively distinguish the nucleotide content of an oligonucleotide, such as standard stringent hybridization, suffers in sensitivity when some fraction of the legitimate product is not eligible for successful detection by that assay.

These discussions suggest two highly desirable traits in any enzyme to be used in the method of the present invention. First, the more rapidly the enzyme executes an entire cleavage reaction, including recognition, cleavage and release, the more signal it may potentially created in the invader-directed cleavage assay. Second, the more successful an enzyme is at focusing on a single cleavage site within a structure, the more of the cleavage product can be successfully detected in a selective read-out. The rationale cited above for making improvements in enzymes to be used in the Invader™-directed cleavage assay are meant to serve as an example of one direction in which improvements might be sought, but not as a limit on either the nature or the applications of improved enzyme activities. As another direction of activity change that would be appropriately considered improvement, the DNAP-associated 5' nucleases may be used as an example. In creating some of the polymerase-deficient 5' nucleases described herein it was found that the those that were created by deletion of substantial portions of the polymerase domain, as depicted in Figure 4, assumed activities that were weak or absent in the parent proteins. These activities included the ability to cleave the non-forked structure shown in Figure 16D, a greatly enhanced ability to exonucleolytically remove nucleotides from the 5' ends of duplexed strands, and a nascent ability to cleave circular molecules without benefit of a free 5' end. These features have contributed to the development of detection assays such as the one depicted in Figure 1A.

In addition to the 5' nucleases derived from DNA polymerases, the present invention also contemplates the use of structure-specific nucleases that are not derived from DNA polymerases. For example, a class of eukaryotic and archaebacterial endonucleases have been identified which have a similar substrate specificity to 5'